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IMMUNOLOGICAL IDENTITY OF RAT LIVER CYTOSOLIC HEME-BINDING PROTEIN WITH PURIFIED AND RECOMBINANT LIVER FATTY ACID BINDING PROTEIN BY WESTERN BLOTS OF TWO-DIMENSIONAL GELS

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We examined the degree of similarity between rat liver cytosolic heme-binding protein (HBP) and rat liver fatty acid binding protein (L-FABP) purified from rat liver cytosol and recombinant L-FABP (rL-FABP). We compared 1) HBP, 2) L-FABP, and 3) rL-FABP prepared in three different laboratories and probed them with three different antisera also from different laboratories on Western blots. The objective was to determine whether the isoform pattern of the recombinant would resemble those of the purified rat liver proteins and whether heme is bound by the isoforms.

To investigate the similarities, we compared the immunoreactivity of purified HBP, L-FABP, and rL-FABP by probing Western blots of 2-D gels with polyclonal antibodies raised against each of these proteins. All of the antibodies react with the same isoelectric species for each of the proteins. In addition, [^{55}Fe]-heme binds equally well to the 2 major HBP isoforms.

Fatty acid-binding proteins are a group of low molecular weight proteins (14-15 kDa) that occur ubiquitously as tissue-specific isoforms (1). The liver fatty acid-binding protein (L-FABP) comprises 2-5% of liver cytosolic proteins (2). The FABPs are considered to have an important role in the transport and metabolism of long-chain fatty acids and possibly that of several other organic anions, including heme (1). A 14 kDa protein was purified from rat liver cytosol and named heme-binding protein (HBP) because it was found to bind heme (in an equimolar complex) with higher affinity, K_d= 0.15 μM , than it bound oleic acid, K_d= 1.5 μM (3). The amino acid

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Abbreviations: L-FABP, liver fatty acid binding protein; rL-FABP, recombinant liver fatty acid binding protein; HBP, heme binding protein; IEF, isoelectric focusing; MW_r, relative molecular weight; NEPHGE, non-equilibrium pH gradient electrophoresis; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; 2-D PAGE, two-dimensional polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

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composition of HBP was found to be identical to that of L-FABP. Western blot analysis of purified rat liver HBP, FABP, and of cytosol, performed on polyacrylamide gels under denaturing conditions (SDS-PAGE), and probed with anti-HBP or anti-L-FABP indicated the similarities between these two proteins. In addition, isoelectric focusing (IEF) and non-denaturing PAGE each revealed only minor differences between HBP, L-FABP, and cytosol, when the blots were probed with anti-HBP (4). In light of these findings, the possibility was considered that HBP is identical to L-FABP. To examine this matter in more detail, we compare here Western blots of two-dimensional gels (2-D PAGE) of rat liver cytosol, purified L-FABP, and HBP. We also determined the immunoreactivity of a recombinant L-FABP, purified from an *Escherichia coli* expression system (7). The blots were probed with antibodies raised against L-FABP and HBP. Regardless of which antibody was used, all proteins showed very similar isoelectric species and the two major HBP isoforms bound [^{55}Fe]-heme.

MATERIALS AND METHODS

Purified proteins and antisera: Purification of HBP from rat liver cytosol was as described in (3). L-FABP was purified as described in (2). Polyclonal antisera against two different preparations of purified L-FABP were raised in rabbits, as described in (5 and 6) and will be designated α-L-FABP$_1$ and α-L-FABP$_2$, respectively. Polyclonal antisera against purified HBP (α-HBP) was raised in rabbits as described in (4). Recombinant L-FABP (rL-FABP) was expressed and purified from *E. coli* as described (7).

Sample preparation: Equal volumes of sample and SDS sample buffer, containing 5% 2-mercaptoethanol were heated at 95° C for 5 min. An 8-fold excess of Garrel's sample buffer (8) was then added.

Gel conditions: Tube gels were prepared as described by O'Farrell (9), for denaturing NEPHGE (10), except that the ampholyte ratio used was 1:2:2 of pH 3.5-10 : 5-7 : 6-8 (Pharmacia). Samples were loaded at the anodic (acidic) end of the gel. Electrophoresis was carried out at 500 V for one hour, followed by 1000 V for 3.5 hr, for a total of 4000 volt hours (Vh). The second dimension SDS-PAGE was run as described previously (11) using a 15% resolving gel.

Western blotting: Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell) for 2 hr at 70 V, with cooling, using 25 mM Tris, 192 mM glycine and 20% methanol as the transfer buffer. The transferred proteins and molecular weight markers were visualized with Ponceau S (12). The blots were destained and dried, prior to blocking for 90 min. in 10 mM Tris HCl, 250 mM NaCl, pH 7.5 (TBS A), containing 5% non-fat dry milk (w/v), 0.1% Nonidet P-40 (w/v), and 0.02% sodium azide (w/v).

Incubation with antibodies: Polyclonal antisera to HBP and to L-FABP$_1$ were diluted 1:500 in blocking solution made with TBS A. Antiserum to L-FABP$_2$ was diluted 1:500 in blocking solution made with 10 mM Tris HCl and 100 mM NaCl, pH 7.5 (TBS B). Incubation was for 2 hr at room temperature. Blots probed with antisera to HBP and L-FABP$_1$ were washed 3 x 10 min with TBS A, containing 0.2% Tween 20 (TTBS A), while blots probed with antisera to L-FABP$_2$ were washed 3 x 10 min with TBS B, containing 0.2% Tween 20 (TTBS B). Blots were then incubated with a 1:3000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad) in blocking buffer for 1 hr and washed 2 x 10 min with TTBS A and 2 x 5 min with alkaline phosphatase buffer (AP buffer): 100 mM Tris HCl, 100 mM NaCl, and 5 mM $MgCl_2$, pH 9.5. Immune complexes were detected using nitro blue tetrazolium/bromochloroindoyl phosphate (U.S. Biochemical) as the substrate for alkaline phosphatase (13).

[^{55}Fe]-heme binding: Purified HBP (28 µg) was incubated with [^{55}Fe]-heme (0.35 mCi/µmole heme), prepared by a modification of the procedure of Adler *et al.* (14) as described in (15), which is approximately equivalent to a 1:1 molar ratio of protein to heme, at 37° C for 30 min. Following incubation, the sample was applied to an Ampholine PAG plate, pH 3.5-9.5 (Pharmacia) and electrophoresed according to the manufacturer's protocol. Additionally, an equal amount of HBP was applied for Coomassie-blue staining and pI marker proteins (Pharmacia) were run for determination of the pH gradient. The lane containing [^{55}Fe]-heme-labeled HBP was excised, dried, and exposed to X-ray film for 2 weeks.

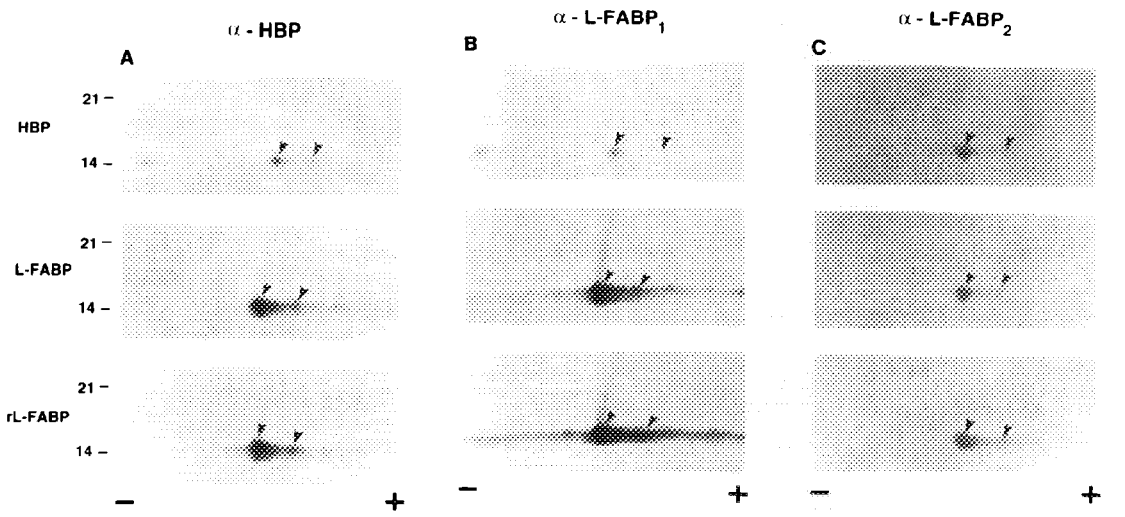

Figure 1. Western blot analysis of 2-D gels of purified HBP and L-FABP and recombinant FABP. Equal amounts (20 μg) of each protein were resolved by 2-D PAGE, transferred to nitocellulose, probed with antisera raised against HBP (α-HBP), L-$FABP_1$ (α-L-$FABP_1$), or L-$FABP_2$, (α-L-$FABP_2$) and visualized colorimetrically, as described in Materials and Methods. The NEPHGE direction is indicated by the + (acid) and - (base) signs at the bottom of each panel. The MW_r of standards (kDa) are indicated to the left . Arrowheads indicate the major, co-migrating isoforms recognized by the antisera.

RESULTS

Figure 1 shows Western blots of 2-D gels of HBP and L-FABP purified from rat liver, and recombinant L-FABP (rL-FABP) purified from *E. coli* probed with anti-HBP (α-HBP), anti-L-$FABP_1$ (α-L-$FABP_1$), or anti-L-$FABP_2$ (α-L-$FABP_2$) antisera. Each antiserum recognized identical immunoreactive species for each antigen. Moreover, species migrating to the same position in the pH gradient are observed with all three antisera and are indicated by arrowheads, although there are differences in overall intensity due to variation in the reactivities of the antibodies. Most informative is the recognition of the rL-FABP isoforms by all 3 antisera. Although this protein was expressed in a procaryotic system, isoforms similar to those detected for the proteins purified from rat liver are present.

In order to verify that the discrete species which are observed for each of the purified proteins are not due to artifacts of isolation, we repeated the experiments above using a rat liver cytosolic extract. Figure 2 demonstrates that the immunoreactive species which are detected by all 3 antisera migrate to similar positions in the pH gradient. These species correspond to those indicated in Figure 1, confirming that procedures used for purification are not responsible for the heterogeneity.

Finally, it is possible that the isoelectric species which we detect are due to varying degrees of fatty acid binding. However, several reports have indicated that fatty acid binding causes rat L-FABP to shift to pI 5.0 (16, 17). Additionally, recent evidence obtained by Börchers and Spener (16) indicates that hematin binds similarly to pI 6.0 and 7.0 isoforms and that oleic acid competes for binding with hematin. These data suggest that heme binding to HBP would be inhibited by the

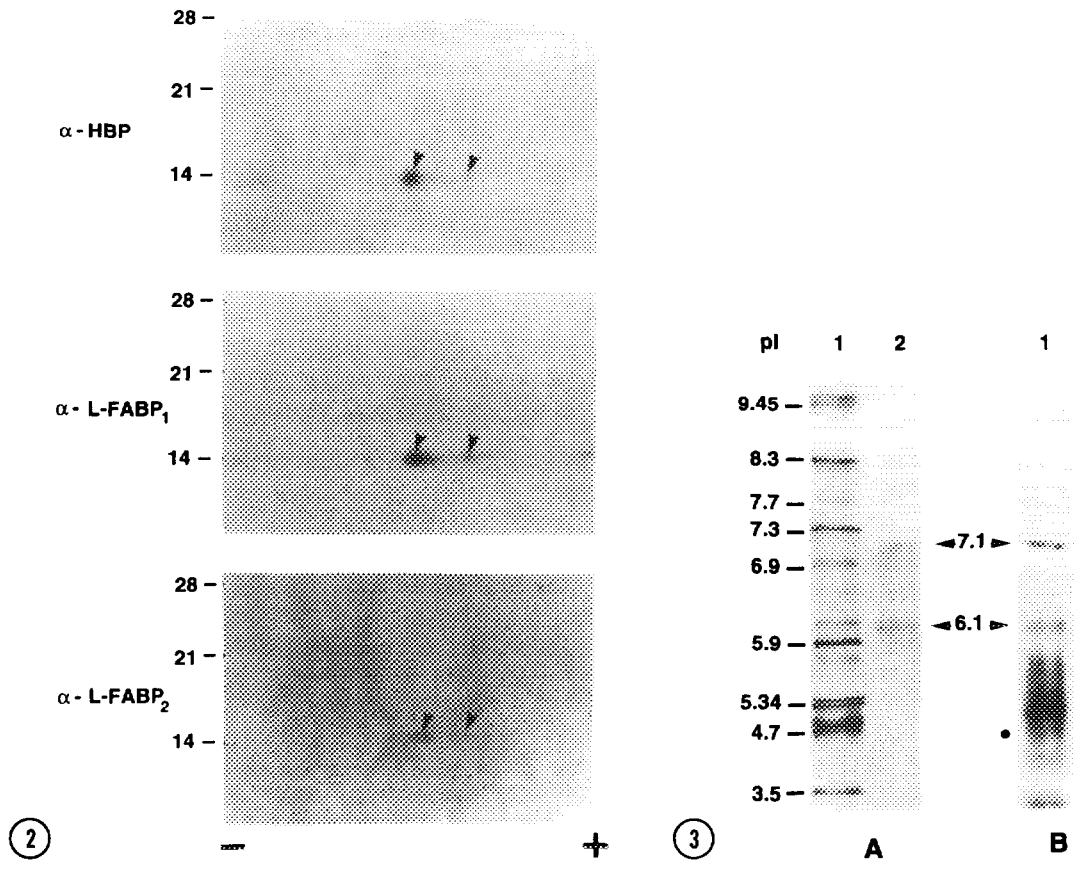

Figure 2. Western blot analysis of 2-D gels of rat liver cytosolic extracts. Rat liver cytosolic extracts (200 μg protein) were prepared as described in Materials and Methods and resolved by 2-D PAGE, transferred to nitrocellulose, probed with antisera, and visualized, as described in Figure 1. The direction of the pH gradient and MW_r are as in Figure 1. Arrowheads indicate the major isoforms recognized by the different antisera. These correspond to the species identified in Figure 1.

Figure 3. [^{55}Fe]-heme binding to HBP isoforms. Non-denaturing isoelectric focusing was performed on HBP (28 μg protein/lane). Figure 3A, lane 1: Coomassie blue-stained isoelectric point markers; lane 2: Coomassie blue-stained HBP; Figure 3B, lane 1: autoradiogram showing the results of [^{55}Fe]-heme binding to HBP isoforms. Arrowheads between A and B indicate the major isoforms which bind [^{55}Fe]-heme and stain with Coomassie blue. The dot indicates the position of sample application. The pH gradient is indicated to the left of Figure 3A, lane 1.

presence of bound fatty acid. Figure 3 shows that purified HBP migrates at pI 6.1 and 7.1 as detected by Coomassie blue staining (Figure 3A, lane 2), and that both of these isoforms bind [^{55}Fe]-heme (Figure 3B, lane 1). These isoforms correspond to the major forms detected by all three antisera in Figures 1 and 2. The dark band at pI 4.7-5.0 (Figure 3B) is probably due to aggregation of unbound [^{55}Fe]-heme and not due to protein isoforms, since there is no Coomassie blue stained band at this position. Finally, [^{14}C]-oleic acid binding causes the pI to shift to 5.0 consistent with the identity of rat L-FABP and HBP (data not shown) and further confirming that the isoforms that we detect in Figures 1 and 2 are not due to varying degrees of fatty acid binding.

DISCUSSION

The data which we have presented confirms and extends the data from several laboratories concerning the identity of rat liver HBP and L-FABP (4, 16). Most importantly, our data demonstrate that recombinant L-FABP behaves similarly to the proteins purified from rat liver.

Western blots of 2-D gels show that antisera raised against HBP and 2 antisera raised against L-FABP recognize the same isoelectric species for each of the purified proteins in Figure 1 and in rat liver cytosol. In other experiments we tried several conditions to obtain reproducible isoelectric focusing. Sample loading at the cathodic end followed by electrophoresis until an equilibrium gradient was established resulted in incomplete entry of the purified proteins into the gel and in poor resolution. Therefore, we used the NEPHGE protocol, in which samples were loaded at the anodic end of the tube gel. This method afforded complete entry of the proteins into the gel and reproducible resolution. However, since a stable pH gradient is not established, the precise isoelectric point of each species can not be determined.

Finally, it can be argued that the multitude of isoelectric species observed are due to artifacts of the NEPHGE procedure. Several points argue against this. The first is that preliminary results of IEF-IEF gels (data not shown) indicated that the number of isoelectric species changed little from performing a second IEF dimension. Second, recent reports address the source of reduced L-FABP isoforms as arising from an asparagine-aspartate exchange at position 105, causing a shift in pI from 7.0 to 6.0, and modification by cysteinylation and by glutathionylation, leading to isoforms with pI's of 7.0/7.1 (16-18). These modifications appear to change the binding stoichiometry and the affinity of L-FABP for ligands, respectively, and suggest that these isoforms may bind preferentailly to a particular ligand depending upon their subcellular localization or the physiological state of the cell (17, 18).

In conclusion, the data presented herein demonstrates that expression of L-FABP in *E. coli* does not appear to detectably alter the protein with respect to its molecular weight or isoelectric forms. Most importantly, the recombinant protein exhibits the same isoforms as L-FABP and HBP purified from rat liver, indicating that modification of the protein is similar. Clearly, this evidence helps validate the use of rL-FABP for binding studies utilizing fatty acids and heme. Mutational analysis of rL-FABP has begun and will allow for further characterization of the amino acid residues which are important for binding of these substrates (19).

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